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Baseline *In Vitro* Activities of the Antimalarials Pyronaridine and Methylene Blue against *Plasmodium falciparum* Isolates from Kenya

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We have analyzed the *in vitro* activities of pyronaridine and methylene blue against 59 *Plasmodium falciparum* isolates from Kenya in association with polymorphisms in *Pfcrt* (codon 76), *Pfmdr1* (codon 86), and *Pfnhe* (full sequence). The median inhibitory concentrations that kill 50% of parasites were 13.5 and 3.3 nM for pyronaridine and methylene blue, respectively. Their activities were not associated with polymorphisms in these genes. The drugs' high *in vitro* activities indicate that they would be efficacious against Kenyan isolates *in vivo*.

Coartem (lumefantrine [LM] and artemether) and amodiaquine (AQ)-artesunate (ART) are currently the first lines of treatment of uncomplicated malaria (8, 29). However, reports indicate that resistance to LM may arise relatively quickly (30). Likewise, evidence suggests that the efficacy of AQ, whose active *in vivo* metabolite is desethylamodiaquine (DEAQ), is reduced in areas of high chloroquine (CQ) resistance (12).

The combinations piperaquine (PIQ)-dihydroartemisinin (DHA) and pyronaridine (PRN)-ART are being developed as alternative antimalarials (2). In spite of these alternatives, the search for new active compounds is being pursued. Methylene blue (MB) is an old antimalarial that was abandoned because of its side effect of turning urine blue. However, this drug has been extensively used for the treatment of methemoglobinemia (5). The burgeoning problem of drug resistance has led to a renewed interest in this drug (28). In this paper, we report on the *in vitro* activities of PRN and MB against *Plasmodium falciparum* field isolates in Kenya and on the change in their activities in relation to polymorphisms in *Pfcrt* at codon 76 (*Pfcrt*-76), in *Pfmdr1* at codon 86 (*Pfmdr1*-86), and in *Pfnhe*.

We analyzed fresh isolates of *Plasmodium falciparum* collected in the Kenyan district of Kilifi and adapted for long-term cultures as detailed previously (13, 15). Antimalarial activity was measured in the presence of various concentrations of each compound, and results were expressed as the drug concentration required for 50% inhibition of [^{3}H]hypoxanthine incorporation into parasite nucleic acid (IC_{50}) (15). We employed two reference strains: V1S, the multidrug-resistant strain, and 3D7, the drug-sensitive strain. We analyzed the antimalarials chloroquine (CQ), MB, AQ, and quinine (QN) (purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom) and LM, PIQ, DEAQ, and DHA (gifts from Steve Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom).

Blood samples (50 μl) of *in vitro*-adapted isolates were spotted onto filter paper, and single-base changes at *Pfcrt*-76 and *Pfmdr1*-86 were detected as reported elsewhere (13). In this paper, we reanalyzed the sequencing of *Pfnhe* published previously (15).

Statistical analyses were carried out using the Stata program (Stata version 11; College Station, TX). We compared differences between groups using the Wilcoxon rank-sum test and measured correlations using the nonparametric Spearman pairwise analysis. All statistical analysis was assessed at the 5% significance level.

We have analyzed the chemosensitivity profiles of 59 *P. falciparum* field isolates against PRN and MB. As comparators, we have included the already-published data on the same isolates against CQ, LM, PIQ and QN, AQ, DEAQ, and DHA (13–15, 27). Median IC_{50}s for PRN and MB against the multidrug-resistant strain V1S were 12 and 1 nM, respectively, and values for the fully sensitive strain 3D7 were 8 and 2 nM, respectively.

Against field isolates, median IC_{50}s for PRN and MB were 13.5 nM (interquartile range [IQR], 4.6 to 31.5) and 3.3 nM (IQR, 1.7 to 8.0), respectively. Values for CQ, LM, PIQ, and DHA are presented in Fig. 1, and those for QN, AQ, and DEAQ were 141.1 nM (IQR, 50.9 to 268.2), 7.8 nM (IQR, 5.4 to 8.6), and 8.5 nM (IQR, 7.4 to 16.9), respectively. PRN was more active than CQ, LM, and PIQ; MB was more active than all these aforementioned drugs, except DHA. Our data are in line with previous reports showing the high potency of PRN *in vitro* against isolates from Cameroon (26), Senegal (22), and Gabon (9).

PRN was used up to the 1990s as a monotherapy for the treatment of malaria in China (4) and was tested in Cameroon with encouraging results (25). Its combination with ART (Pyramax) has proven efficacious in many African countries, including Kenya (24, 31), in line with our *in vitro* data. PRN has also been proven potent *in vitro* against *P. falciparum* isolates from Southeast Asia, a known area of multidrug resistance (23). Interestingly, PRN is also active against *Plasmodium vivax in vitro* (23), and recently, the *in vivo* efficacy of PRN-ART against *P. vivax* has been shown, making this combination a potential drug for the treatment of *P. vivax* infection as well (18).

MB has proven efficacious in African countries, mainly in Burkina Faso (3, 10, 11, 32), and has gametocytocidal properties; thus, it could be part of treatment combinations to reduce transmission of *P. falciparum* (6). Our data show that MB is active *in vitro*, in line with 2 previous studies using African parasites (1, 17).

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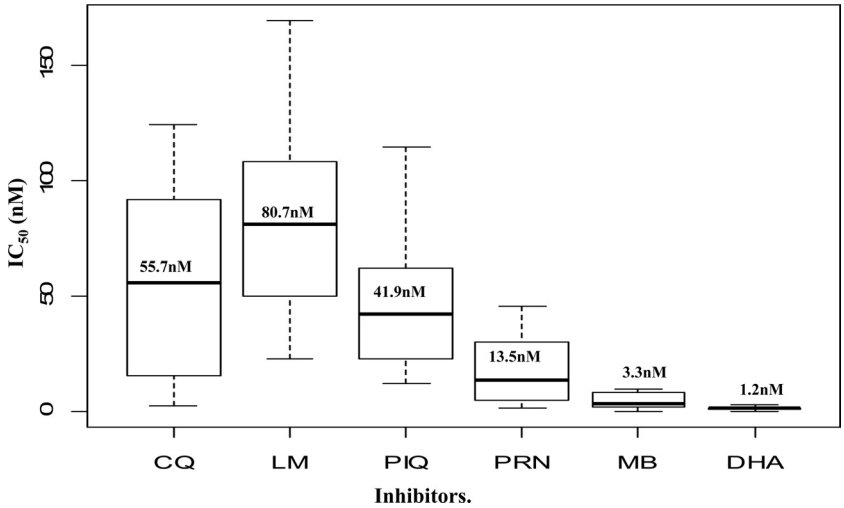

FIG 1 Median inhibitory concentrations that kill 50% of parasites (IC_{50}s) of chloroquine (CQ), lumefantrine (LM), piperaquine (PIQ), pyronaridine (PRN), methylene blue (MB), and dihydroartemisinin (DHA). Values are nM, and median IC_{50}s are represented in bold. Parasites were adapted *in vitro* for long-term culture prior to assessment of IC_{50}s.

Thus, this drug would also be efficacious against the Kenyan parasite population.

We also investigated the role of polymorphisms within *Pfcrt*-76, *Pfmdr1*-86, and *Pfnhe*. PRN is more active against parasites harboring the wild-type sequence than against those harboring the mutant sequence at the *Pfcrt*-76 codon (IC_{50}s of 6 versus 20 nM) and at the *Pfmdr1*-86 codon (IC_{50}s of 7 versus 19 nM); however, these differences were not significant ($P > 0.05$). This lack of association has also been reported elsewhere (19).

Data for MB showed that its activity was not affected by polymorphisms in *Pfcrt*-76 and *Pfmdr1*-86. Likewise, no change was observed between the activity of PRN and MB and polymorphisms in *Pfnhe*, a gene associated with QN resistance (16), in line with a previous report (17).

We also analyzed the correlation between PRN and MB *in vitro* activities and those of CQ, LM, PIQ, AQ, DEAQ, QN, and DHA. PRN activity was significantly correlated with those of the quinolone-based drugs PIQ, AQ, DEAQ, and QN but not with CQs (Table 1). PRN *in vitro* activity was found to correlate with CQ, AQ, or QN in some studies (20, 21, 23) but not in others (9, 19). We did not find a correlation between PRN and DHA activities, as reported previously (9). However, a significant correlation between PRN and ART was found (19, 23). Since artemisinin resistance is now emerging (7), it is important to establish the extent to which this resistance may affect PRN activity. We observed a significant correlation between MB and CQ, LM, DEAQ, QN, and DHA, while no association was found between MB and PIQ and between QN and PRN. To the best of our knowledge, only one study addressed this correlation: MB activity was not correlated with CQ, QN, DEAQ, LM, and DHA (17). Clearly, further investigations are needed to define the relationship between the *in vitro* activities of MB and other antimalarials.

We have provided the first evidence of the *in vitro* activity of PRN and MB against isolates from Kenya. The high *in vitro* activities of these 2 drugs are in line with data reported in other parts of Africa and also confirm (at least in the case of PRN) the reported efficacy of PRN-ART in Kenya.

TABLE 1 Correlation coefficient (*r*) between the *in vitro* activities of PRN and MB and those of CQ, LM, PIQ, AQ, DEAQ, QN, and DHA[a]

	PRN		MB	
Drug	Spearman *R* value	*P* value	Spearman *R* value	*P* value
CQ	0.3	0.090	0.37	**0.010**
LM	−0.07	0.642	0.40	**0.005**
PIQ	0.33	**0.023**	0.20	0.179
AQ	0.58	**<0.001**	0.20	0.182
DEAQ	0.46	**0.001**	0.26	0.079
QN	0.35	**0.016**	0.70	**<0.001**
DHA	0.20	0.184	0.53	**<0.001**
PRN			0.23	0.127
MB	0.23	0.130		

[a] The nonparametric Spearman statistical test was used, and values in bold are those that are statistically significant.

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